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In Vitro Study of Ethanolic Extract of *Hypericum perforatum* L. on Growth and Sporulation of Some Bacteria and Fungi*

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Abstract: The antibacterial activity of an ethanolic extract of *Hypericum perforatum* L. was investigated against 8 Gram-negative bacteria (*Pseudomonas fluorescens, Pseudomonas phaseolicola, Pseudomonas glycinea, Erwinia carotovora, Enterobacter cloacae, Klebsiella pneumoniae, Agrobacterium tumefaciens, Azotobacter chrococcum*) and 2 Gram-positive bacteria (*Bacillus mycoides, Bacillus subtilis*) by the disc-diffusion and broth tube dilution methods. Ethanol was used for extraction of the plant. The species *Pseudomonas glycinea* and *Azotobacter chrococcum* showed extreme sensitivity to the extract of *H. perforatum*, while no effect was observed on bacteria *Klebsiella pneumoniae*. Minimum inhibitory concentration (MIC) of the ethanolic extract varied between 1.25 and 3.5 mg/ml. The antifungal activity of the *H. perforatum* extract at concentrations of 5-45 mg/ml was determined by the method of spore counting; a concentration of 45 mg/ml showed the greatest fungistatic activity. In the case of inoculation of 1 x 10^2 spores of fungi, the number of spores was decreased: with *Fusarium oxysporum*, only 5 spores were identified, and with *Penicillium canescens*, 15 spores.

Key Words: *Hypericum perforatum*, antibacterial activity, antifungal activity

Hypericum perforatum'un Etonolik Özütlerinin Bazı Bakteri ve Fungi Sporulasyonuna Etkisi Üzerine In vitro Çalışma

Özet: *Hypericum perfaratum* L.'nın etanolik özütünün antimikrobiyal aktivitesi sekiz adet Gram negatif bakteri (*Pseudomonas florescent, Pseudomonas phaseolicola, Pseudomonas glycinea, Erwinia caratovora, Entrobacter cloaceae, Klebsiella pneumoniae, Agrobacterium tumefacies, Azotobacter chrococcum*) ve iki Gram pozitif bakteri üzerinde (*Bacillus mycoides, Bacillus subtilis*) disk difüzyon ve sıvı tüp seyreltme yöntemleri ile araştırılmıştır. Bitkiden özüt eldesi için etanol kullanılmıştır. *Pseudomonas glycinea* ve *Azotobacter chrococcum* türleri *H. perforatum* özütüne yüksek duyarlılık gösterirken, *Klebsiella pneumoniae* üzerine herhangi bir etkisi olmamıştır. Etanolik özütün minimal engelleyici derişimi (MIC) 1,25-3,5 mg/ml. arasında değişmektedir. *H. perforatum* özütünün antifungal aktivitesi 5-45 mg/ml. derişimlerde spor sayma yöntemi ile belirlenmiş ve 45 mg/ml.'lik derişim en büyük fungistatik aktivite göstermiştir. 1 x 10^2 fungi sporunun aşılanması ile spor sayısı azalmıştır; *Fusarium oxysporum*'da sadece 5 spor belirlenirken *Penicillium canescent* ile 15 spor belirlenmiştir.

Anahtar Sözcükler: *Hypericum perforatum*, antibakteriyal aktivite, antifunfal aktivite

Introduction

In recent years, the consumption of *Hypericum perforatum* L. *(Hypericaceae = Clusiaceae*)-derived products has increased dramatically and it is presently one of the most consumed medicinal plants in the world (1). The *H. perforatum*-derived products are available as phytopharmaceuticals and nutraceuticals, teas, tinctures, juices, and oily macerates (2). *H. perforatum* has a wide range of medicinal applications, including skin wounds, eczema, burns, diseases of the alimentary tract, and psychological disorders (3).

H. perforatum ethanolic extracts contain many phenolic compounds (hypericin, hyperforin and their derivatives, rutin, hyperoside, quercetin, chlorogenic acid, flavonols and flavones), suggesting that they could have important antioxidant properties (4).

Hypericin has shown antibacterial, antiviral and anti-inflammatory activity (5) and hyperforin is the main compound involved in antidepressant activity (6). Hyperforin exhibits effects against the methicillin-resistant strains of *Staphylococcus aureus* with a minimum inhibitory concentration (MIC) value of 1.0 µg/ml (7).

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H. perforatum is used both for therapeutic purposes and as a flavoring in the preparation of foods and alcoholic beverages. In addition, infusion, alcoholic tincture, and fluid extract of plant are used in the flavoring industry for preparing liqueurs, especially digestive and tonic bitters (8).

The aim of the study was to determine the level of antimicrobial activity of ethanolic extract of *H. perforatum* toward some bacteria and fungi.

Materials and Methods

Plant material

The aerial parts (only flowers) of *H. perforatum (Hypericaceae = Clusiaceae)* were collected in the region of Kragujevac, central Serbia in June 2006. The plant material was air-dried at room temperature (20 ± 2 °C). The identity and purity were determined by Dr V. Stevanovic, Department of Botany, Faculty of Biology, University of Belgrade. A voucher specimen (voucher 0799HP) of the plant is deposited in the Herbarium of the Department of Botany, Faculty of Biology, University of Belgrade, Serbia.

Reparation of the extract

The air-dried *H. perforatum* (40 g) was broken into small pieces 2-6 mm by using a cylindrical crusher, and extracted with ethanol: water solution (80:20) (180 ml) using a Soxhlet apparatus. The mixture was filtered through a paper filter (Whatman, No.1) and evaporated. The residue (5.8 g) was stored in a dark glass bottle for further processing.

Test microorganisms

The fungi *Fusarium oxysporum* (Schlecht) and *Penicillium canescens* (Sopp) were isolated from fruits (citrus, orange) in the process of deterioration. The fungi were reseeded on potato-glucose agar, on which they developed for 7 days at room temperature of 20 ºC under alternating day-night light conditions. They were reseeded on a new potato-glucose substrate, on which they developed for another 7 days. The reseeding procedure was performed four times, after which the pure cultures needed for determination were obtained.

Test bacteria used in this experiment were: *Bacillus mycoides* (IPH), *Bacillus subtilis* (IPH), *Pseudomonas fluorescens* (B28), *Pseudomonas phaseolicola* (B29), *Pseudomonas glycinea* (B40), *Erwinia carotovora* (B31), *Enterobacter cloacae* (B22), *Klebsiella pneumoniae* (B26), *Agrobacterium tumefaciens* (B11), and *Azotobacter chrococcum* (B14).

The tested bacteria cultures were obtained from the Institute for Health Protection (IPH) and the Faculty of Agriculture, University of Belgrade, Serbia. The Laboratory for Microbiology, Department of Biology, Faculty of Science, University of Kragujevac, Serbia confirmed identification of all tested microorganisms (B11-40).

Antibacterial activity of ethanolic extract obtained from *H. perforatum*

The antibacterial activity of the ethanolic extract of the whole plant of *H. perforatum* was investigated by the disc-diffusion method on nutrient agar (9,10). It was performed using a 24 h culture at 37 ºC reseeded on nutrient broth. The cultures were adjusted to 5.6 x 10^6 CFU/ml with sterile water. One milliliter of the suspensions was added over the plates containing nutrient agar to get a uniform microbial growth on both control and test plates. The extract of *H. perforatum* was dissolved in 96% ethanol (100 mg/ml) and sterilized. Under aseptic conditions, empty sterilized discs (Whatman no. 5, 14 mm diameter) were impregnated with 250 µl, 100 µl, 50 µl, 25 µl of different concentrations (25 mg/disc, 10 mg/disc, 5 mg/disc, 2.5 mg/disc) of the respective extract and placed on the agar surface. The plates were left for 30 min at room temperature to allow the diffusion of extract, and then were incubated at 37°C. After the incubation period (48 h), the zones of inhibition were measured and presented in mm. The paper disc of solvent (ethanol) was used as a control. Sinacilin (12.5 mg/disc) was used as the standard antibiotic for comparison. Each test was performed in triplicate.

The MIC of the ethanolic extract was determined by a routine in vitro procedure (11). Briefly, the extract was tested by the microdilution two-fold serial technique. The extract of *H. perforatum* was dissolved in a minimum quantity of 5% DMSO (20 mg/ml, 15 mg/ml). A series of two-fold dilutions of the extract, ranging from 0.12 mg/ml to 10 mg/ml, was prepared in Mueller-Hinton broth (12) with the addition 0.1 ml of bacterial spore suspension (5.4x10^6 CFU/ml). The presented results were determined after 24 hours, and the MICs were

determined as the lowest concentration of extract inhibiting visible growth of each organism on the agar (13,14). Experiments were carried out in triplicate. A control test was also performed containing inoculated broth supplemented with 5% DMSO only at the same dilutions used in our experiments. Sinacilin (1 mg/ml) was chosen as the control drug.

Antifungal activity of the ethanolic extract of *H. perforatum*

The antifungal activity of 5-45 mg/ml plant extract (in phosphate buffer, pH=8) was investigated by the method of spore counting (Nauber's chamber, hemocytometer) (15). The 0.2 ml inoculum of the fungi researched ($1x10^2$ CFU/ml spores) was added to each sample of the broth with the extract. The presented results were determined after 24 hours.

Results and Discussion

Results of antimicrobial activity of ethanolic extract of *H. perforatum* are shown in Table 1 and Figure 1.

H. perforatum extracts have a broad-spectrum antimicrobial activity against some bacteria and fungi, including *Staphylococcus aureus, Staphylococcus mutans, Proteus vulgaris, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans* (16-19).

Recently, it has been reported that the methanolic extract of *H. perforatum* is active in vitro against *Arcobacter* and *Staphylococcus* species and contains chlorogenic acid, caffeic acid, rutin, hyperoside, quercetin and hypericin as the main compounds responsible for its antimicrobial activity (20,21).

We have examined the influence of ethanolic extract of *H. perforatum* against some bacteria and fungi grown on fruits and vegetables. It was aimed that these results would provide an answer about the possibility of using the extract of *H. perforatum* as the native antibacterial protector.

The antibacterial activities (disc-diffusion and microdilution methods) of the ethanolic extract of *H. perforatum* against the bacteria are presented in Table 1. The results show that ethanolic extract of *H. perforatum*

Table 1. Antibacterial activity of ethanolic extract of *H. perforatum*.

Microorganism	Diameter of zones of inhibition (mm)a,b,c					MIC (mg/ml)a,b,c	
	Mass of extract, standard/disc				Sinacilin	Extract	Sinacilin
	25 mg	10 mg	5 mg	2.5 mg	12.5 mg		
Pseudomonas fluorescens (B28)	8 ± 1	1 ± 1	0.5 ± 1	/	16 ± 1	1.25	0.675
Pseudomonas phaseolicola (B29)	6 ± 1	2 ± 0.5	0.5 ± 1	/		1.25	
Pseudomonas glycinea (B40)	8.5 ± 1	1.5 ± 1	1 ± 0.5	/		2.50	
Erwinia carotovora (B31)	6 ± 0.5	1.5 ± 1	0.5 ± 1	/		1.25	
Enterobacter cloacae (B22)	7.5 ± 1	1.5 ± 1	0.5 ± 1	/		2.50	
Klebsiella pneumoniae (B26)	5 ± 0.5	1 ± 1	0.5 ± 1	/		3.50	
Agrobacterium tumefaciens (B11)	8 ± 0.5	2.5 ± 1	0.5 ± 1	/		2.50	
Azotobacter chrococcum (B14)	8.5 ± 1	2 ± 1	1 ± 1	/		2.50	
Bacillus mycoides (IPH)	6.5 ± 1	2 ± 1	2 ± 0.5	/	27.5 ± 1	3.50	0.016
Bacillus subtilis (IPH)	7 ± 1	1 ± 1	0.5 ± 1	/		3.5	

[a] mean value ± SD, n = 3 [the zone of inhibition (in mm) not including disc of 14 mm in diameter].

[b] Values are the mean of three replicates using 5×10^6 of each culture.

[c] Solvents controls (ethanol, DMSO) were negative.

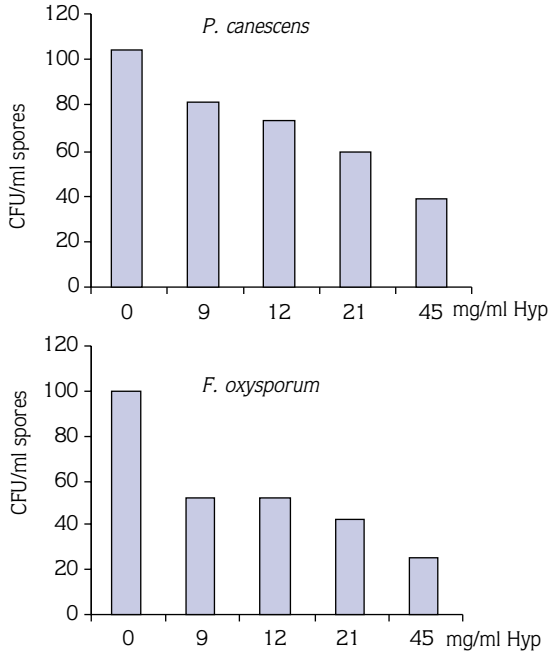

Figure 1. Antifungal activity of ethanolic extract of *H. perforatum* L.

has antibacterial activity against all 10 investigated bacteria, and the greatest inhibition was observed with 25 mg extract per disc. The diameter inhibition zones ranged from 5 mm to 8.5 mm (not including the diameter of the disc) with the high zone values observed against *P. glycinea* (8.5 mm) and *A. chrococcum* (8.5 mm). Doses of 5-10 mg extract per disc showed smaller zones of inhibition, in the range of 1-2.50 mm. The lowest concentration of 2.5 mg extract per disc showed no inhibitory effect.

The extract of *H. perforatum* showed the least inhibitory effect on bacteria *K. pneumoniae.* The concentration of 25 mg extract per disc showed inhibition of 5 mm. The extract concentration of 5 mg/disc showed an inhibition of 0.5 mm.

Antibacterial activity of ethanolic extract of *H. perforatum* depends on bacteria type, both Gram-positive and Gram-negative. MIC of ethanolic extract of *H. perforatum* varied between 1.25-3.5 mg/ml.

The results of the antifungal activity of the ethanol extract are shown in Figure 1. The number of spores of fungi decreased with increasing concentration of ethanolic extract. In accordance with this fact, the sample with the concentration of 45 mg/ml ethanolic extract showed the highest fungistatic activity, with a decrease in the number of spores to 5 spores for *F. oxysporum* and to 15 spores for *P. canescens*, in the case of inoculation with 1 x 10^2 CFU/ml spores.

Conclusion

On the basis of the results, it was concluded that *H. perforatum* extract showed antibacterial activity against all 10 investigated bacteria. The level of antibacterial activity is a function of the investigated concentration. The ethanolic extract of *H. perforatum* showed strong antibacterial activity against *P. glycinea.* Inhibition zones were measured from 1 to 8.5 mm in the presence of 5-25 mg of the extract per disc.

The least level of activity of ethanolic extract of *H. perforatum* was shown on the bacteria *K. pneumoniae* in the presence of 25 mg of a plant extract per disc (level of inhibition: 5 mm).

The lowest extract mass of 2.5 mg per disc did not show antibacterial activity against any of the 10 investigated bacteria. MIC was established by dilution method and ranged from 1.25-3.50 mg/ml for all tested bacteria.

Antifungal activity of *H. perforatum* extract against the fungi *P. canescens* and *F. oxysporum* was demonstrated to decrease the number of spores by 62% and by 72%, respectively.

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